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Dysregulation of Toll-Like Receptor 7 Compromises Innate and Adaptive T Cell Responses and Host Resistance to an Attenuated West Nile Virus Infection in Old Mice

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ABSTRACT

The elderly are known to have enhanced susceptibility to infections and an impaired capacity to respond to vaccination. West Nile virus (WNV), a mosquito-borne flavivirus, has induced severe neurological symptoms, mostly in the elderly population. No vaccines are available for human use. Recent work showed that an attenuated WNV, a nonstructural (NS) 4B-P38G mutant, induced no lethality but strong immune responses in young (6- to 10-week-old) mice. While studying protective efficacy, we found unexpectedly that old (21- to 22-month) mice were susceptible to WNV NS4B-P38G mutant infection but were protected from subsequent lethal wild-type WNV challenge. Compared to responses in young mice, the NS4B-P38G mutant triggered higher inflammatory cytokine and interleukin-10 (IL-10) production, a delayed γδ T cell expansion, and lower antibody and WNV-specific T cell responses in old mice. Toll-like receptor 7 (TLR7) is expressed on multiple types of cells. Impaired TLR7 signaling in old mice led to dendritic cell (DC) antigen-presenting function compromise and a reduced γδ T cell and regulatory T cell (Treg) expansion during NS4B-P38G mutant infection. R848, a TLR7 agonist, decreased host vulnerability in NS4B-P38G-infected old mice by enhancing γδ T cell and Treg expansion and the antigen-presenting capacity of DCs, thereby promoting T cell responses. In summary, our results suggest that dysregulation of TLR7 partially contributes to impaired innate and adaptive T cell responses and an enhanced vulnerability in old mice during WNV NS4B-P38G mutant infection. R848 increases the safety and efficacy during immunization of old mice with the WNV NS4B-P38G mutant.

IMPORTANCE

The elderly are known to have enhanced susceptibility to infections and an impaired capacity to respond to vaccination. West Nile virus (WNV), an emerging mosquito-borne flavivirus, has induced severe neurological symptoms more frequently in the elderly population. No vaccines are available for human use. Here, we used an aged mouse model to investigate the protective efficacy of an attenuated WNV, the nonstructural 4B-P38G mutant, which was previously shown to induce no lethality but strong immune responses in young adult mice. Studies that contribute to a mechanistic understanding of immune defects in the elderly will allow the development of strategies to improve responses to infectious diseases and to increase vaccine efficacy and safety in aging individuals.

Longer life expectancy has increased the proportion of the elderly worldwide. According to the United Nations' population division, the number of people worldwide aged 60 and older is expected to reach more than one billion by 2020. Meanwhile, age-associated immunosenescence has contributed to both enhanced susceptibility to infectious diseases and impaired capacity to respond to vaccination in the geriatric population. Currently, the mechanisms that lead to the decline in immunity are not clearly understood.

West Nile virus (WNV), a mosquito-borne flavivirus, has induced acute viral encephalitis and neurological sequelae in North America for the past decade (1). One major risk factor for fatality due to WNV infection in humans is aging (1–3). Although multiple approaches have been taken in WNV vaccine research, none have been approved for human use. Development of safe and effective vaccines, in particular for the potentially susceptible elderly population, remains a high priority. The WNV genome is a single-stranded, positive-sense RNA molecule, about 11,000 nucleotides in length, that is translated and processed into 10 proteins: three structural proteins (envelope [E], membrane, and nucleocapsid) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (4, 5). The NS4B protein of flaviviruses is known to be associated with evasion of host immune responses and viral replication (6–9). The NS4B P38 residue is conserved among mosquito- and tick-borne flaviviruses. Previous work has identified an attenuated mutant with a P38G substitution in NS4B protein by utilizing site-directed mutagenesis of a

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WNV New York 1999 strain (NY99) infectious clone. Two other compensatory mutations, NS4B-T116I and NS3-N480H, were also found upon full genome sequencing of the mutant (10). The WNV NS4B-P38G mutant was shown to display multiple suitable features for an ideal vaccine candidate in young adult mice: significantly reduced neuroinvasiveness, induction of greater innate cytokine and T cell responses than wild-type WNV, and protection of mice from subsequent lethal wild-type WNV infection (11). More recently, we have shown that WNV NS4B-P38G mutant infection in human monocytic and macrophage cells induced robust cell intrinsic innate cytokine responses, which is a further indication of utility of this mutant as a potential vaccine candidate in humans (12).

We and others have previously defined an aged mouse model to study WNV pathogenesis and protective immunity (13, 14). In the present study, we investigated the protective efficacy of the WNV NS4B-P38G mutant in immunization of old (21- to 22-month-old) mice. The mutant induced levels of recall responses in old mice that were similar to those in young mice, and it protected them from subsequent lethal wild-type WNV challenge. Unexpectedly, the mutant induced about 30% lethality during initial challenge in old mice. In investigating the underlying immune mechanisms, we found that an impaired Toll-like receptor 7 (TLR7) signaling in old mice led to the dysfunction of innate and adaptive T cell responses to the NS4B-P38G mutant, which together contributed to an enhanced host susceptibility upon initial challenge.

TABLE 1 Quantitative PCR gene array

Gene symbol	Gene description
Casp8	Caspase 8
Ccl2	Chemokine (C-C motif) ligand 2
Ccl5	Chemokine (C-C motif) ligand 5
Cxcl10	Chemokine (C-X-C motif) ligand 10
Ddx58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
Ifi27l2a	Interferon alpha-inducible protein 27-like 2A
Ifi44	Interferon-induced protein 44
Ifih1	Interferon-induced protein with helicase C domain 1
Ifit1	Interferon-induced protein with tetratricopeptide repeats 1
Ifit2	Interferon-induced protein with tetratricopeptide repeats 2
Ifitm3	Interferon-induced transmembrane protein 3
Il10	Interleukin-10
Il17a	Interleukin-17A
Il18	Interleukin-18
Irf3	Interferon-regulatory factor 3
Irf5	Interferon-regulatory factor 5
Irf7	Interferon-regulatory factor 7
Irf9	Interferon-regulatory factor 9
Isg15	ISG15 ubiquitin-like modifier
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1
Oas1a	2′-5′ oligoadenylate synthetase 1
Rsad2	Radical *S*-adenosylmethionine domain containing 2
Stat1	Signal transducer and activator of transcription 1
Tgfb1	Transforming growth factor β1
β-Actin	Beta-actin

MATERIALS AND METHODS

Mice. Six- to 10-week-old C57BL/6 (B6) OT II transgenic and 21- to 22-month-old B6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and the National Institute of Aging (Bethesda, MD), respectively. TLR3$^{-/-}$ mice (B6 background) were obtained from M. Diamond (Washington University, St. Louis, MO). TLR7$^{-/-}$ mice (VG 143, B6 × 129 F_2 background; Regeneron Inc., Tarrytown, NY) were bred to the B6 background by backcrossing for 7 to 10 successive generations (15–17). the WNV NS4B-P38G mutant was produced by utilizing site-directed mutagenesis and passaged twice in Vero cells (10). The parental strain WNV NY99, a kind gift from R. Tesh, was passaged once in Vero cells and twice in C6/36 cells. Mice were inoculated intraperitoneally (i.p.) with 1,500 PFU of the WNV NS4B-P38G mutant. In some experiments, mice were injected i.p. with 30 or 50 μg of R848 (InvivoGen, San Diego, CA) at 4 h before WNV infection (18). Mice were rechallenged with a 100% lethal dose (LD_{100}) (2,500 PFU) of WNV at day 30 after primary infection. Infected mice were monitored twice daily for signs of morbidity. All animal experiments were approved by the Animal Care and Use Committee at the University of Texas Medical Branch.

Infection and stimulation of cells. Bone marrow (BM)-derived dendritic cells (DCs) or macrophages were generated as described previously (19). Briefly, BM cells were cultured for 6 days in medium supplemented with granulocyte-macrophage colony-stimulating factor and interleukin-4 (IL-4) or macrophage colony-stimulating factor (Peprotech) to generate myeloid DCs or macrophages and were infected with WNV at a multiplicity of infection (MOI) of 0.1 or 0.02, respectively. Supernatants and cells were collected at 24 h and 96 h postinfection to measure cytokine production. For stimulation, BM-derived DCs or macrophages (2×10^5 cells/well) were cultured at 37°C in RPMI 1640 or Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) in 96-well plates with 1 μg/ml of CL097 or 20 μg/ml of poly(I·C) (InvivoGen). Supernatants and cells were harvested at 24 h to measure cytokine production. In some experiments, cells were treated with R848 (20 μg/ml) for 4 h before WNV infection.

Focus-forming assay (FFA) for WNV titer. Vero cell monolayers were incubated with sample dilutions first for 1 h. A semisolid overlay containing 0.8% methylcellulose (Sigma-Aldrich, St. Louis, MO), 3% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine (Invitrogen) was then added. At 48 h, the semisolid overlay was removed, and cell monolayers were washed with phosphate-buffered saline (PBS), air dried, and fixed with a 1:1 acetone-methanol solution for at least 30 min at −20°C. Cells were next subjected to immunohistochemical (IHC) staining with a rabbit WNV polyclonal antibody followed by goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (KPL, Gaithersburg, MD) at room temperature for 1 h. After secondary antibody treatment, cells were incubated with a peroxidase substrate (Vector Laboratories, Burlingame, CA) until color developed. The number of foci was determined and used to calculate viral titers, expressed as focus-forming units (FFU) per milliliter. The limit of detection for the assay was 90 FFU/ml.

Quantitative PCR (qPCR). WNV-infected samples were resuspended in TRIzol (Invitrogen) for RNA extraction. cDNA was synthesized by using a qScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The sequences of the primer sets for WNV envelope, TLR7, and cytokine cDNAs and PCR conditions were described previously (20–22). The assay was performed in the CFX96 real-time PCR system (Bio-Rad). Gene expression was calculated based on either threshold cycle (C_T) values by using the formula $2^{-[CT(\text{target gene}) - CT(\text{GAPDH gene})]}$ as described before (23) or the ratio of the amount of amplified gene to the amount of β-actin cDNA as the relative levels in each sample.

PCR arrays. A 25-gene PrimePCR assay (Bio-Rad) was performed following the manufacturer's protocol. Briefly, RNA was purified from noninfected and WNV-infected cells by using an RNeasy extraction kit (Qiagen, Valencia, CA) and quantitated by spectrometry. cDNA was synthesized by using iScript family reverse transcription kits and then loaded onto 96-well PCR array plates for amplification on the CFX96 real-time PCR system (Bio-Rad). A list of 25 genes is provided in Table 1. Data analysis was performed by using CFX manager software. Four genes were selected as reference genes.

Cytokine Bioplex. Culture supernatants or mouse sera were collected for analysis of cytokine production by using a Bio-Plex Pro mouse cytokine assay (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with recombinant WNV-E protein (24) overnight at 4°C at 100 ng/well. Sera were diluted 1/30 in PBS with 2% bovine serum albumin (BSA) and incubated for 1 h at room temperature. Alkaline phosphatase-conjugated goat anti-mouse IgG or IgM (Sigma-Aldrich) at a dilution of 1/1,000 in PBS-Tween was then added and left for 1 h. Color was developed with *p*-nitrophenyl phosphate (Sigma-Aldrich) and intensity determined at an absorbance of 405 nm by using a spectrophotometer.

Histology and IHC staining. Mice were transcardially perfused with PBS. Spleens and brains were removed, placed in 4% paraformaldehyde (PFA), and left overnight at 4°C, followed by 70% ethanol for 24 h before embedding in optimal cutting temperature compound. Samples were then prepared for hematoxylin and eosin (H&E) and IHC staining at the Histopathology Laboratory at the University of Texas Medical Branch (UTMB). For IHC staining, a fast red chromogen kit (Biopath Laboratories, Oklahoma City, OK) was used. Rabbit anti-WNV sera (1:250 dilution) and anti-rabbit IgG alkaline phosphatase (AP)-labeled antibody (1:500; KPL) were used as primary and secondary antibodies.

Flow cytometry. Splenocytes were stained with antibodies for CD11c, CD80, CD86, CD3, T cell receptor δ (TCRδ), CD4, or CD8 (e-Biosciences, San Diego, CA). To measure intracellular cytokine production, splenocytes were stimulated with WNV-specific NS3 and E peptides (RRWCFD GPRTNTILE and PVGRLVTVNPFVSVA, respectively [25]) for CD4 T cells or WNV-specific NS4B and E peptides (SSVWNATTA and IALTF LAV, respectively [26, 27]) for CD8 T cells for 5 h at 37°C. Golgi-plug (BD Biosciences) was added at the beginning of stimulation. Cells were stained with antibodies for CD3, CD4, or CD8, fixed in 2% paraformaldehyde, permeabilized with 0.5% saponin before addition of anti-gamma interferon (anti-IFN-γ), anti-tumor necrosis factor alpha (anti-TNF-α), or control rat IgG1 (e-Biosciences), and examined by using a C6 flow cytometer (Accuri Cytometers, Ann Arbor, MI). Foxp3 expression was analyzed by using a kit from eBioscience according to the manufacturer's instructions. Data were analyzed by using CFlow Plus (Accuri Cytometers).

***In vitro* T cell priming assays.** $CD4^+$ T cells and DCs were purified from splenocytes of naive OT II transgenic mice and WNV-infected mice, respectively, by using anti-CD11c and anti-CD4 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). $CD4^+$ T cells (2×10^6 cells) were labeled with 0.5 μmol/liter carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen), and mixed with DCs at a 10:1 ratio with or without OVA residues 323 to 339 (1 μg/ml; GenScript Corporation, Piscataway, NJ), and left for 5 days. Cells were harvested, and $CD4^+$ T cells were gated for analysis of proliferation. Supernatant was collected at day 3 for analysis of cytokine production.

Statistical analysis. Survival curve comparisons were performed using Prism software (GraphPad Software, San Diego, CA) statistical analysis, which uses the log rank test (equivalent to the Mantel-Haenszel test). Values for viral burden, cytokine production, and antibody and T cell response experiments are presented as means ± standard errors of the means (SEM). *P* values for these experiments were calculated with a nonpaired Student *t* test. Statistical significance was accepted at a *P* value of <0.05.

RESULTS

Old mice are more vulnerable to WNV NS4B-P38G mutant infection than young mice but are protected from subsequent lethal wild-type WNV challenge. WNV NS4B-P38G, a highly attenuated mutant, was previously reported to induce strong immune responses in young adult mice (11, 17). To investigate its protective efficacy in old mice, we infected 21- to 22-month-old mice by the i.p. route with 1,500 PFU of the WNV NS4B-P38G mutant. Virus-infected young adult mice were used as controls. All young mice survived infection for at least 30 days. Surprisingly, 33% of the old mice succumbed to infection (Fig. 1A). About 40% of NS4B-P38G mutant-infected old mice developed severe neurologic signs within 2 weeks, including hunched posture, weight loss, and repetitive behaviors, whereas no clinical signs were observed in virus-infected young mice (data not shown). Compared to that in young mice, there was about 3-fold-higher viremia in old mice on day 3 (Fig. 1B). No significant differences were noted in viral load in spleens between the two groups of mice on days 3, 7, and 13 (Fig. 1C). In the brain, there was a 45-fold increase in viral load in old mice on day 13 by qPCR assay (Fig. 1D). WNV antigen was also detected in neurons of the cerebral cortex, corpus striatum, hippocampus, and brainstem of old mice that succumbed to infection on day 13 but was not detected in the same areas of noninfected old mice or virus-infected young mice (Fig. 1E). Necrosis was not observed in the spleens in either group before or after WNV infection (Fig. 1F). Pathological examination of brains of old mice that succumbed to infection on day 13 revealed perivascular cuffing by mononuclear infiltrates in the corpus striatum, the hippocampus (Fig. 1G), penetrating vessels in neocortex, and leptomeninges (data not shown), whereas cellular infiltrates were not noted in virus-infected young mice or noninfected old mice (Fig. 1G). Thus, higher viremia and elevated viral load and inflammatory responses in the brain contributed to enhanced vulnerability in old mice during primary infection with the NS4B-P38G mutant. On day 30, surviving mice of both groups were rechallenged with a lethal dose of wild-type WNV NY99 and were monitored daily for mortality. All mice survived secondary WNV infection with no obvious clinical signs within a 1-month monitoring period (data not shown). Overall, although the NS4B-P38G mutant protects old mice from wild-type WNV challenge, it is virulent in these mice and induces a lethal infection in a proportion of mice during initial infection.

The NS4B-P38G mutant induces a higher innate cytokine production and impaired T and B cell responses in old mice. To investigate the underlying mechanisms of enhanced susceptibility of old mice to WNV NS4B-P38G mutant infection, we next examined immune responses in these mice. Type 1 interferons (IFNs), including IFN-α and IFN-β, participate in direct control of WNV dissemination and clearance (28). Higher peripheral proinflammatory cytokine and interleukin-10 (IL-10) levels have been associated with increasing viral load and brain pathology (21, 29, 30). We measured serum cytokine production on days 3 and 6 postinfection. Compared to young mice, old mice produced higher levels of proinflammatory cytokines, including IL-1β, IL-17, and TNF-α, on day 3, and IL-10 production was elevated in old mice at both time points (Table 2). No differences in type 1 IFN responses were noted between the two groups (data not shown). γδ T cells are important innate lymphocytes that expand quickly in response to wild-type WNV infection and protect the host from lethal encephalitis (31). Both the cell number and the percentage of γδ T cells in young mice were increased on day 3, whereas these cells did not expand in old mice until day 6 postinfection (Fig. 2A and B).

B cell-mediated humoral immune responses are critical for the host defense against disseminated infection by wild-type WNV (32, 33). As shown in Fig. 2C and D, WNV-specific IgM and IgG production was diminished in old mice on day 8. No differences were noted between the two groups on days 3, 6, and 21 postinfection, except that IgG levels were lower in old mice on day 4 after secondary challenge with wild-type WNV.

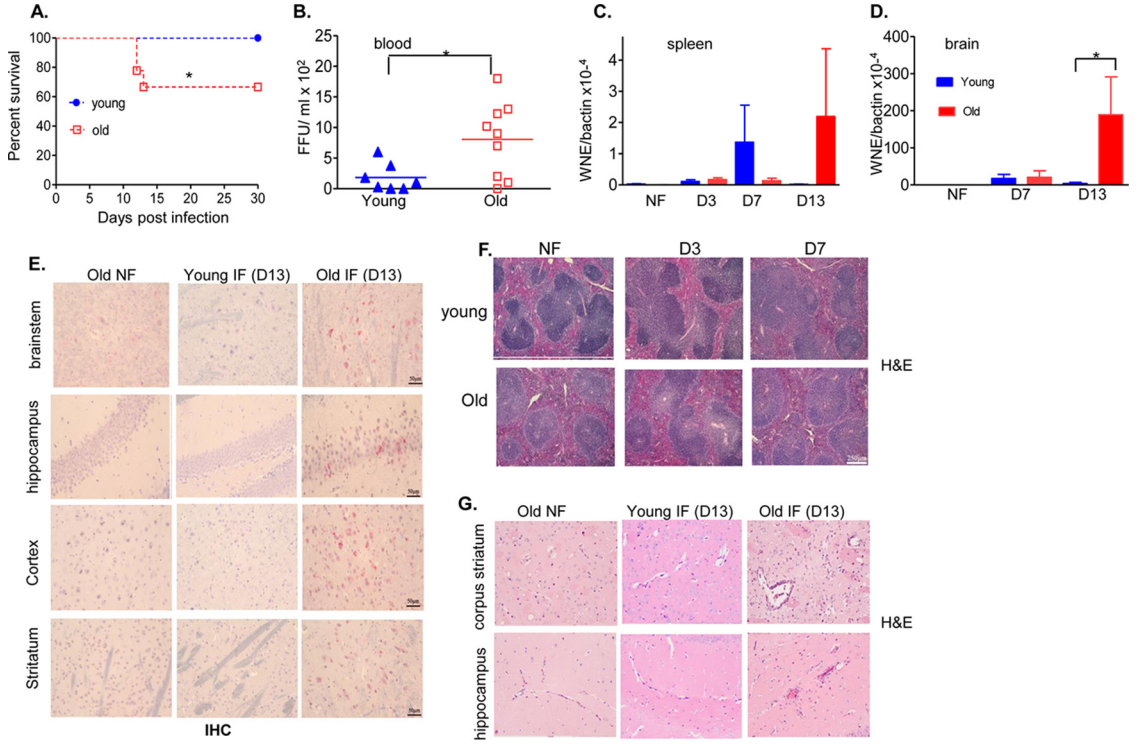

FIG 1 Old mice are vulnerable to WNV NS4B-P38G mutant infection. (A) Survival of young and old mice after i.p. injection with the WNV NS4B-P38G mutant ($n = 14$ and $n = 9$ for young and old mice, respectively). (B) The viral load in blood was determined by using FFA at day 3 postinfection. (C and D) The viral loads in spleen and brain were determined by qPCR. Data shown are representative of three independent experiments. (E to G) IHC staining for WNV antigen (E) and H&E staining (F and G) The representative images (magnification, ×20) shown are from brains and spleens from noninfected (NF) mice and WNV-infected (IF) mice at the indicated time points. *, $P < 0.05$ compared to young mice.

CD4$^+$ and CD8$^+$ T cells contribute to long-lasting protective immunity during wild-type WNV infection (34, 35). As shown in Fig. 2E and F, CD4$^+$ and CD8$^+$ T cells in old mice produced much less IFN-γ than those in young mice on day 8. The percentage of IFN-γ$^+$ TNF-α$^+$ CD8$^+$ T cells was also lower in old mice than in young mice (Fig. 2G and H). Furthermore, CD4$^+$ T cells of old mice produced less IL-2 upon *ex vivo* stimulation with WNV E and NS3 peptides (Fig. 2I). During secondary challenge with lethal wild-type WNV, CD4$^+$ and CD8$^+$ T cells of old mice had responses either similar to or higher than the cells of young mice (Fig. 2E to I). In summary, our results suggest that the NS4B-P38G mutant induced a higher inflammatory cytokine and IL-10 production, a delayed γδ T cell expansion, and lower WNV-specific antibody and T cell responses in old mice during primary infection than those seen in young mice. Further, humoral and T cell responses remained unaffected in NS4B-P38G-immunized old mice during secondary infection with wild-type WNV.

Impaired TLR7 signaling in old mice mainly contributes to reduced innate cytokine responses in NS4B-P38G-infected DCs. DCs represent the most important antigen-presenting cells (APC) exhibiting the unique capacity to initiate T cell responses. An impaired innate cytokine response has been reported in WNV-infected DCs of elderly donors (36). Mouse DCs are permissive to WNV infection. To determine DC functions in old mice, we first

TABLE 2 Serum cytokine levels at days 3 and 6 postinfection

	Level, pg/ml (mean ± SEM)[a]			
	Day 3		Day 6	
Cytokine	Young mice	Old mice	Young mice	Old mice
IL-1β	279.8 ± 43.9	522.6 ± 51.1*	417.2 ± 96.8	307.5 ± 28.7
IL-6	12.5 ± 1.6	14.2 ± 2.3	9.8 ± 6.6	2.9 ± 1.8
IL-12p40	322.3 ± 20.4	515.2 ± 105.3	225.1 ± 37.8	455.2 ± 102.8
IL-17	83.84 ± 18.5	187.5 ± 34.9*	126.0 ± 45.9	49.8 ± 6.5
TNF-α	290.7 ± 48.8	562.3 ± 68.6*	478.9 ± 84.2	341.6 ± 38.6
IFN-γ	18.4 ± 3.2	24.4 ± 5.4	3.8 ± 1.3	0.90 ± 0.90
IL-10	80.7 ± 6.5	141.4 ± 6.2**	83.4 ± 23.7	179.1 ± 10.6*

[a] Blood was harvested from WNV-infected mice at the indicated time points postinfection. Serum cytokine levels were measured with Bioplex. *, $P < 0.05$; **, $P < 0.01$ (compared to young group).

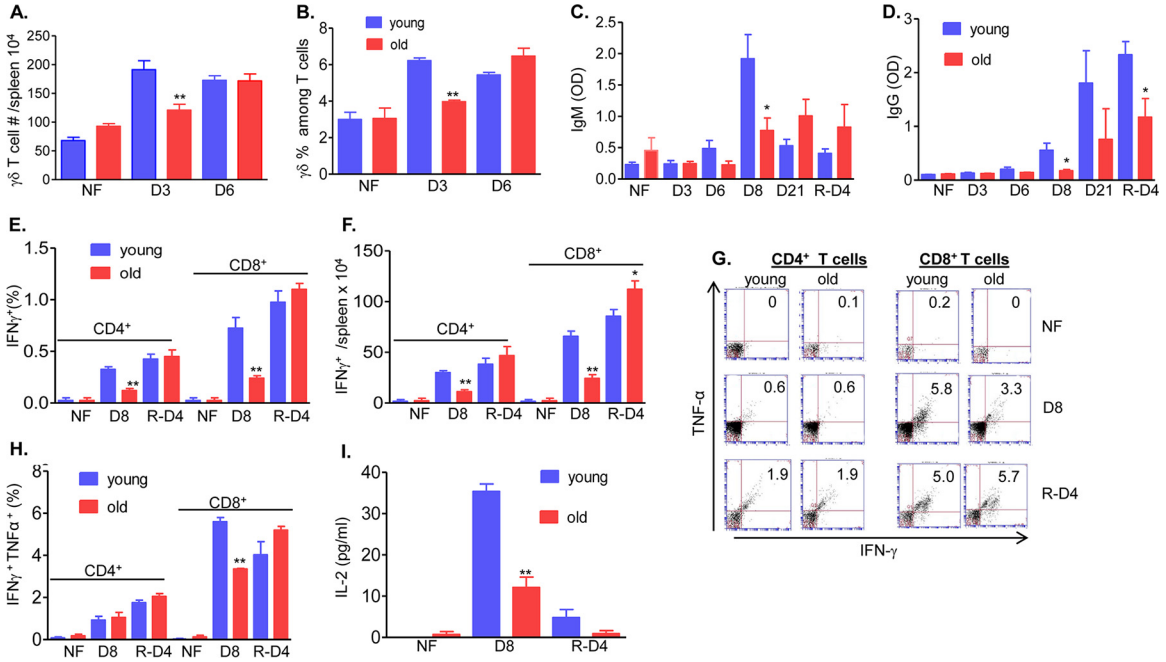

FIG 2 Immune responses to WNV NS4B-P38G mutant infection in old mice. (A and B) Splenocytes were isolated before infection (NF) and on days (D) 3 and 6 and stained for TCRγδ and CD3. (A) Total number of γδ T cells per mouse. (B) Total $\gamma\delta^+$ percentage among splenic T cells ($n = 3$ to 6). Data shown are representative of two independent experiments. (C and D) Humoral responses during primary and secondary WNV infection. Sera were collected from noninfected (NF) mice or mice infected with the WNV NS4B-P38G mutant at days 3, 6, 8, and 21 after primary infection and day 4 after rechallenge (R-D4) with wild-type WNV. The development of WNV-specific IgM (C) or IgG (D) antibodies was determined by ELISA. Data are presented as means ± SEM ($n = 4$ to 8, pooled from 2 separate experiments). (E and F) Splenocytes were harvested at days 0 and 8 after primary WNV NS4B-P38G infection or day 4 after rechallenge with wild-type WNV and were cultured *ex vivo* with WNV peptides for 5 h and stained for IFN-γ, TNF-α, and T cell markers. (E) Percent positive IFN-γ^+ cells. (F) Total number of IFN-γ^+ T cell subsets per spleen. (G and H) Percentage of IFN-γ^+ TNF-α^+ among $CD4^+$ or $CD8^+$ T cells. Cells were gated on $CD4^+$ or $CD8^+$ T cells. Panel G shows a representative image. (I) IL-2 production in cell culture supernatant. **, $P < 0.01$; *, $P < 0.05$ (compared to young mice); $n = 4$ or 5 mice/group from two separate experiments.

performed a WNV-inducible gene PCR array. As shown in Fig. 3A, compared to that in DCs from young mice, IL-10 was upregulated in NS4B-P38G-infected DCs from old mice, whereas interferon-stimulated genes (ISG) and chemokine genes were either up- or downregulated in old DCs compared to young DCs, indicating an age-related differential gene expression. These differences were not due to viral replication as evidenced by there being no difference in viral load between NS4B-P38G-infected young and old DCs (Fig. 3B). Pathogen recognition receptors (PRRs), including TLR3, TLR7, and retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs), such as RIG-I and melanoma differentiation antigen 5 (MDA-5), are involved in WNV recognition and trigger the signaling cascade leading to the production of type 1 IFNs and proinflammatory cytokines (15, 21, 37, 38). Upregulation of TLR3 and TLR7 gene expression due to NS4B-P38G mutant infection was delayed and/or reduced in old DCs compared to young DCs. Nevertheless, RIG-I and MDA-5 gene expression was either mildly increased or not changed in old DCs (Fig. 3C). In further investigating this, we found that either stimulation with CL097, a TLR7/8 agonist, or WNV NS4B-P38G mutant infection induced IL-1β, IL-6, and TNF-α production and upregulation of IFN-β gene expression in young DCs, but these effects were compromised in old DCs (Fig. 3D to G). In contrast, no differences in induction of the above-mentioned genes were noted between young and old DCs following stimulation with poly(I·C), a TLR3 agonist (Fig. 3D to G). IL-10 expression levels were similar in poly(I·C)- and CL097-treated young and old DCs (Fig. 3H). To verify these results, we next infected DCs of wild-type and $TLR3^{-/-}$ mice with the NS4B-P38G mutant. Similar levels of IL-1β, IL-6, TNF-α, and IL-10 were induced in DCs of both groups of mice (Fig. 3I to K and M), except that IFN-β expression was diminished in infected $TLR3^{-/-}$ DCs compared to the wild-type group (Fig. 3L). In contrast to the case for DCs, CL097 stimulation triggered more IL-6 and TNF-α production in macrophages of old mice than in the young group. No differences in inflammatory cytokine production were detected in macrophages isolated from young and old mice following either poly(I·C) stimulation or WNV NS4B-P38G mutant infection, though there was a higher IL-10 production following NS4B-P38G infection in old macrophages (Fig. 4). Furthermore, WNV infection boosted innate cytokine production in wild-type and $TLR3^{-/-}$ macrophages, with no significant differences between the two groups (data not shown). Taken together, these results indicate that an impaired TLR7 signaling mainly contributed to lower innate cytokine responses in DCs, but not in macrophages, of old mice during NS4B-P38G mutant infection.

TLR7 agonist reduces host susceptibility to NS4B-P38G mutant infection in old mice by increasing γδ T cell and regulatory T cell (Treg) expansion and enhancing DC antigen-presenting capacity, thereby promoting the T cell response. To further investigate the role of TLR7 in host protection in old mice, R848 was administered by the i.p. route prior to WNV NS4B-P38G mutant infection. As shown in Fig. 5A, all R848-treated old mice survived NS4B-P38G mutant infection with no obvious clinical signs

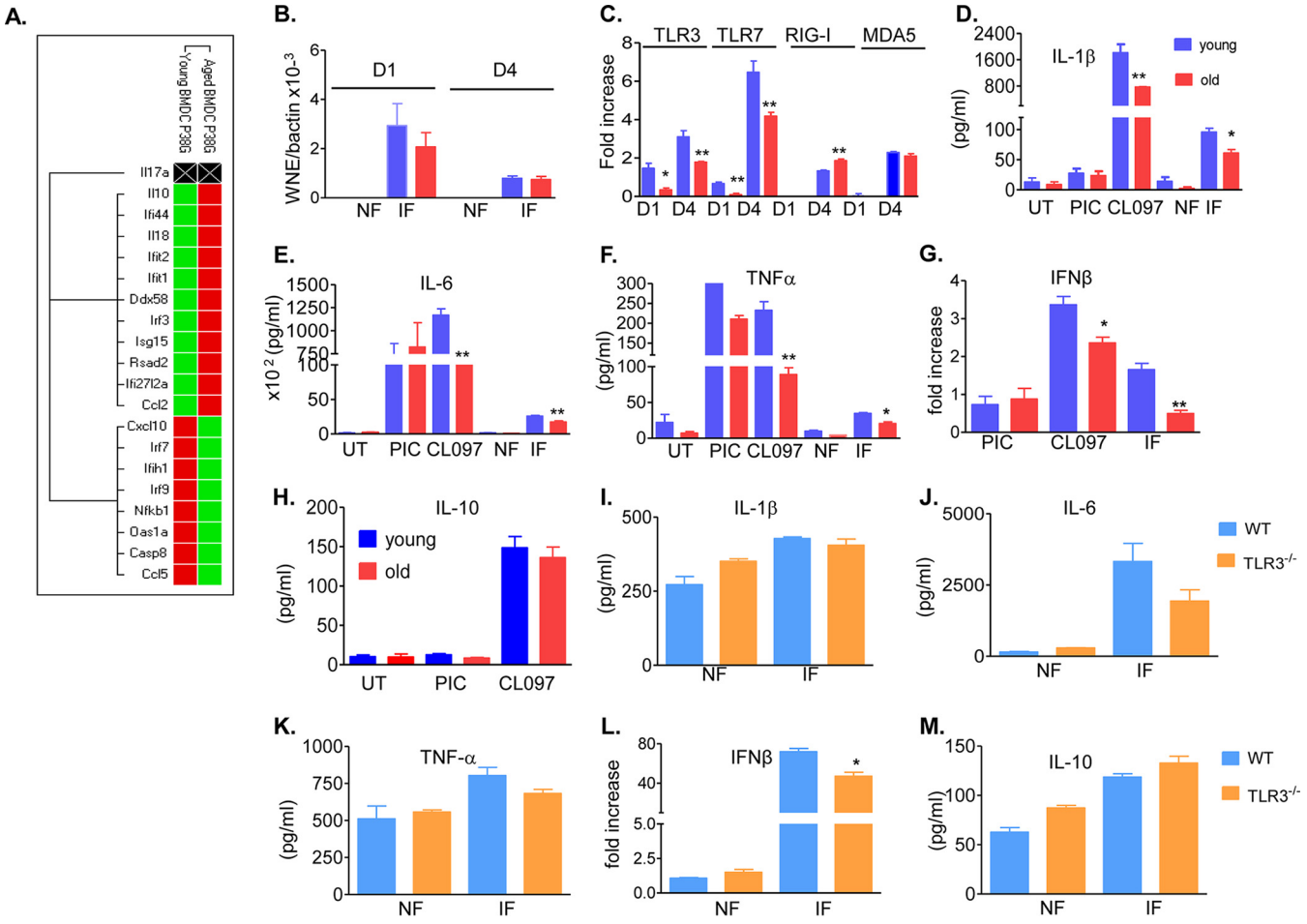

FIG 3 WNV NS4B-P38G mutant infection and TLR agonist stimulation in BM-derived DCs. (A to H) Young and old DCs were infected with the WNV NS4B-P38G mutant or TLR agonists harvested at indicated time points. (A) PrimePCR array, presented as a clustergram image which depicts relative expression of a sample as follows: upregulation (higher expression), red squares; downregulation (lower expression), green squares; no regulation, black squares. On the outer edges of the data plot is a dendrogram which indicates the clustering hierarchy. (B and C) Viral load (B) and PRR gene expression (C) were measured at the indicated time points by qPCR. (D to H) Cytokine production was determined by using Bioplex (D to F and H) or by qPCR (G). (I to M) DCs of wild-type and TLR3$^{-/-}$ mice were infected with the WNV NS4B-P38G mutant. Cytokine production was determined by using Bioplex or qPCR at the indicated time points. For panels G and L, data are presented as the fold increase compared to mock-infected cells. Results are representative of three experiments ($n = 3$ to 6). **, $P < 0.01$; *, $P < 0.05$ (compared to the young group).

within a 1-month period. Viremia in R848-treated old mice was diminished on day 3 postinfection compared to that in controls (Fig. 5B). There was a reduced viral load in the spleens of treated old mice compared to controls on day 3, but the differences became insignificant at later time points (Fig. 5C). We also noted a lower viral load in the brains of treated mice on day 13 compared to that in control group (Fig. 5D). Similar levels of germinal center proliferation were observed in spleens of R848-treated naive and infected old mice on days 3 and 7, and these were also comparable in the spleens of control mice. There was no spleen necrosis observed in either group (Fig. 1F and 5E). Brain tissues, including cerebral cortex (Fig. 5F), cerebellum, corpus striatum, brain stem, and hippocampus (data not shown), from R848-treated and WNV-infected old mice were examined on day 13. No inflammation was found in any of the mice. Thus, R848 increases host resistance against primary NS4B-P38G mutant infection in old mice. Surviving mice of both groups were next rechallenged with a lethal dose of wild-type WNV on day 30 and were monitored daily for morbidity. Both R848-treated old mice and controls had 100% survival rates during secondary wild-type WNV infection (data not shown). To determine the effect of R848 on host immunity in old mice, we measured innate cytokine production in sera on day 3 and found no differences in IL-1β, IL-6, IL-17, TNF-α, and IL-10 production between treated and nontreated old mice, except that IL-12p40 levels were reduced by 50% in the R848-treated group (data not shown). R848 was previously shown to boost γδ T cell expansion in young mice upon WNV infection (39). Here, we found that R848 induced a 40% increase in γδ T cells in old mice on day 3 compared to that in the nontreated control group, and this was same as those observed in young mice (Fig. 6A). No differences in WNV-specific IgM and IgG responses were noted between the R848-treated and control old mice on days 3 and 32 following secondary wild-type WNV infection, though there was a trend of a lower IgM response in the treated group ($P = 0.05$) on day 8 during primary NS4B-P38G mutant infection (Fig. 6B and C).

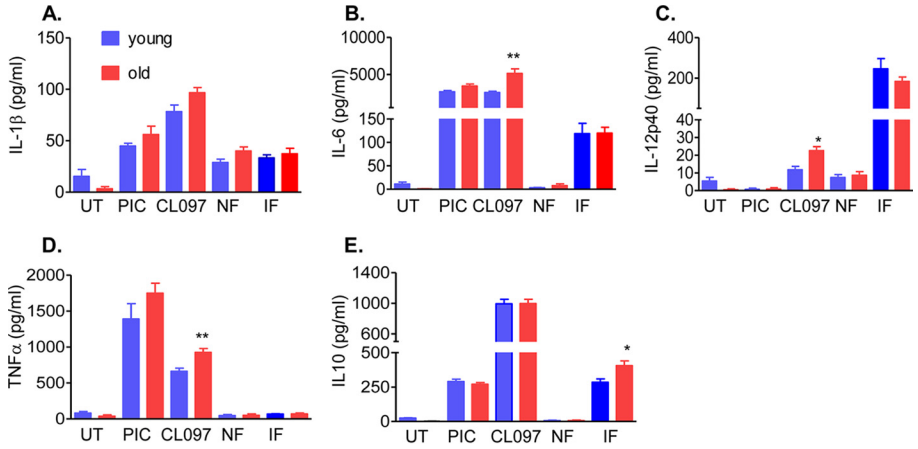

FIG 4 WNV NS4B-P38G mutant infection or TLR3 and TLR7 agonist stimulation in BM-derived macrophages. Cytokine production was determined by using Bioplex. Results are representative of two experiments ($n = 3$ to 6). **, $P < 0.01$; *, $P < 0.05$ (compared to the young group).

We have recently demonstrated that TLR7-dependent MyD88 signaling is required for T cell priming during NS4B-P38G mutant infection of young adult mice (17). As shown in Fig. 7A, R848-treated old mice had an elevated $CD4^+$ T cell expansion on day 8 postinfection. There was also more IL-2 and IFN-γ production in splenocytes from R848-treated old mice than in those from the nontreated control group (Fig. 7B). During secondary challenge with lethal wild-type WNV, R848-treated old mice had increased numbers of $CD4^+$ and $CD8^+$ T cells and produced similar or higher levels of IL-2 and IFN-γ upon *ex vivo* stimulation compared to the control group (Fig. 7A and C). To investigate the mechanism by which R848 regulates T cell responses, we first determined whether R848 promoted T cell priming via enhancing DC APC functions. Old DCs were stimulated with R848 *in vitro*, followed by WNV NS4B-P38G infection. Compared to that in the nontreated control group, R848 significantly elevated IL-1β, IL-6, IL-12, and TNF-α production in old DCs during WNV NS4B-

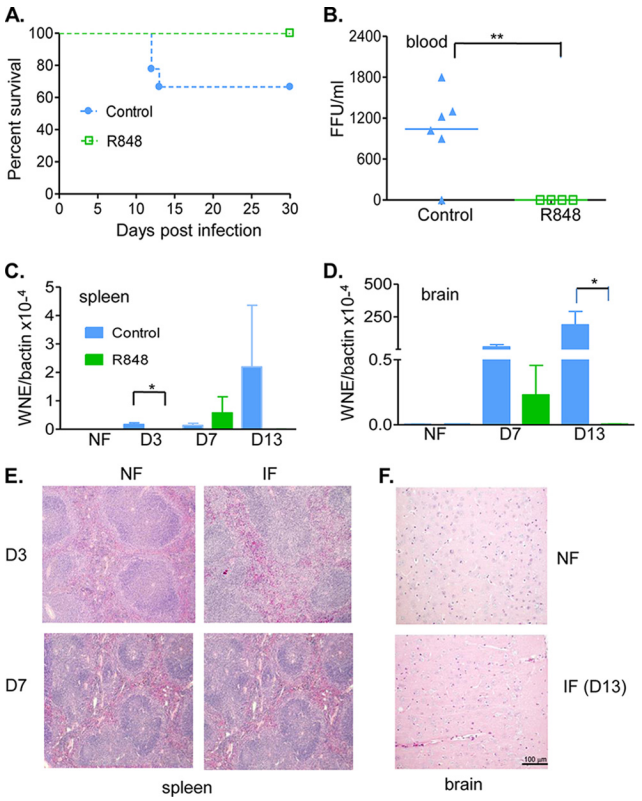

FIG 5 R848 increases host resistance against WNV NS4B-P38G mutant infection in old mice. (A) Survival of R848-treated and mock old mice after i.p. injection with the WNV NS4B-P38G mutant ($n = 9$ and $n = 7$ for control and R848-treated mice, respectively). (B) The viral load in blood was determined by using FFA on day 3. (C and D) The viral loads in spleen and brain were determined by qPCR. (E and F) H&E staining. The representative images (magnification, ×20) shown are from spleens and brains from WNV-infected (IF) mice and noninfected (NF) R848-treated mice at the indicated time points. **, $P < 0.01$; *, $P < 0.05$ (compared to the mock-treated group).

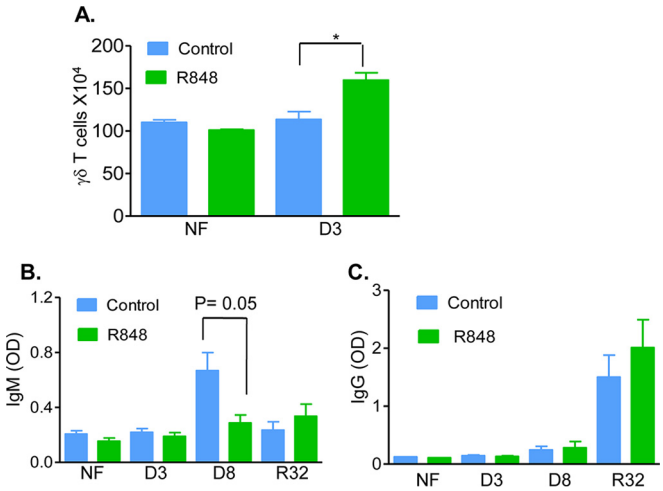

FIG 6 γδ T cell and antibody responses to WNV NS4B-P38G mutant infection in R848-treated old mice. (A) Splenocytes were isolated before infection (NF) and on day 3 (D3) postinfection and stained for TCRγδ and CD3. The total number of γδ T cells per mouse is shown. (B and C) Humoral response during primary and secondary WNV infection. Sera were collected from noninfected (NF) mice or mice infected with the WNV NS4B-P38G mutant at days 3 and 8 during primary infection and day 32 after rechallenge (R-32) with wild-type WNV. WNV-specific IgM (B) or IgG (C) antibodies were determined by ELISA. Data are presented as means ± SEM $n = 4$ to 9, pooled from 2 or 3 separate experiments. *, $P < 0.05$ compared to mock-treated group.

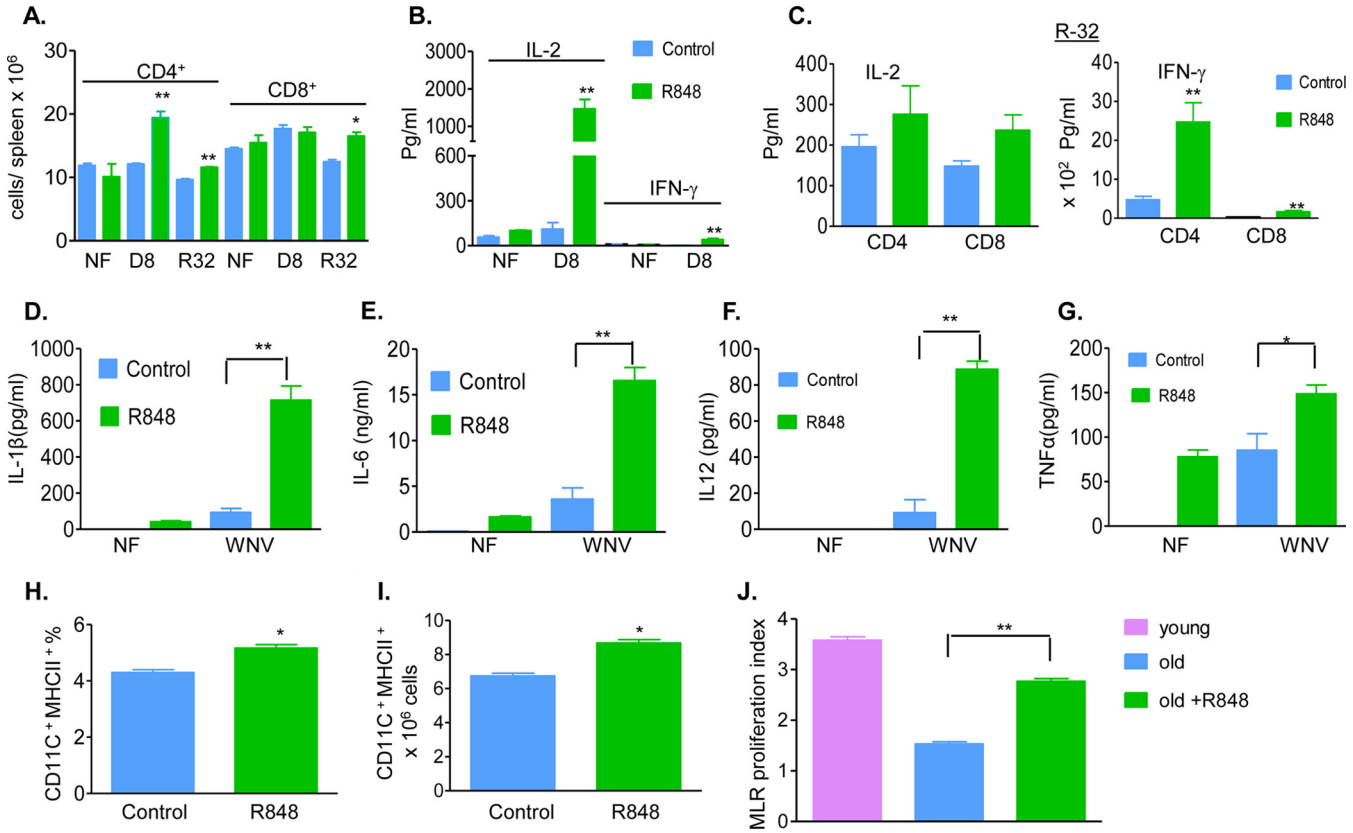

FIG 7 R848 enhances the WNV-specific T cell response to NS4B-P38G mutant infection. (A to C) Splenocytes were harvested on days 0 and 8 after primary WNV NS4B-P38G infection or on day 32 after rechallenge with wild-type WNV. (A) Total number of T cell subsets per spleen. (B and C) IL-2 and IFN-γ production in culture supernatant of splenocytes cultured alone (B) or with WNV-specific peptides for $CD4^+$ or $CD8^+$ T cells (C). (D to G) BM-derived DCs of old mice were treated with R848 for 4 h, followed by WNV NS4B-P38G mutant infection. Cytokine production on day 4 postinfection was measured by Bioplex. (H and I) MHC II expression on DCs of mock- or R848-treated old mice on day 3 postinfection by percentage (H) or cell number (I). (J) Mixed-lymphocyte reaction (MLR) assay. CFSE-labeled $CD4^+$ T cells from naive OT II transgenic mice were cocultured with DCs from WNV-NS4B-P38G mutant-infected young or mock-or R848-treated and NS4B-P38G-infected old mice in the presence or absence of OVA residues 323 to 339. Cells were harvested at day 5 and analyzed for T cell proliferation ($n = 5$) *, $P < 0.05$; **, $P < 0.01$ (compared to mock-treated group).

P38G mutant infection (Fig. 7D to G). On day 3 postinfection, $CD11c^+$ DCs of R848-treated old mice displayed a higher level of major histocompatibility complex (MHC) II expression by both percentage and cell number than those of the control group (Fig. 7H and I). Finally, a mixed-lymphocyte reaction (MLR) assay showed that DCs of R848-treated old mice had a greater T cell priming capacity than did those of the nontreated group and had even reached levels similar to those of DCs of NS4B-P38G-infected young mice (Fig. 7J).

Accumulating evidence shows that immune suppression increases with age (40, 41). Therefore, we investigated whether R848 decreased the immunosuppressive effects on T cells in old mice. R848-treated old mouse splenocytes produced levels of IL-10 either higher than or same as those in young mice during *ex vivo* culture alone or upon stimulation with WNV-specific T cell peptides (Fig. 8A). WNV NS4B-P38G mutant infection induced $CD4^+$ Treg expansion in young adult mice on day 7, whereas $CD4^+$ Treg expansion was compromised in NS4B-P38G-infected old mice and $TLR7^{-/-}$ mice. R848 treatment in old mice triggered Treg expansion to levels similar to those in young mice on day 7 (Fig. 8B). Myeloid-derived suppressor cells (MDSCs) expand during inflammation and infection and are known to have a remarkable ability to suppress T cell responses (42). While R848 did not affect MDSC expansion in old mice, it decreased the number of MDSCs in NS4B-P38G mutant-infected young mice (Fig. 8C). Thus, R848 either did not affect or slightly increased the immunosuppressive cytokine and cellular responses in old mice during infection. These results suggest that R848 enhances effector T cell responses in old mice by improving DC APC functions, thereby promoting T cell priming, but not via decreasing the immunosuppressive effects on T cells.

DISCUSSION

The poor response to vaccination in the elderly population has been a major hurdle in protecting the elderly from infectious diseases. Consequently, multiple immunizations, often with large amounts of antigen, are needed to induce protective immunity. No WNV vaccines are available for human use. Development of safe and effective vaccines remains a high priority for WNV vaccine development. Recent phase II clinical trials of Chimeri-Vax-WNV02, a chimeric WNV vaccine candidate, demonstrated comparable safety in all age groups. However, differences in neutralizing antibody titers and viremia were reported in treatment of different age groups, though no reports of statistical analysis are yet avail-

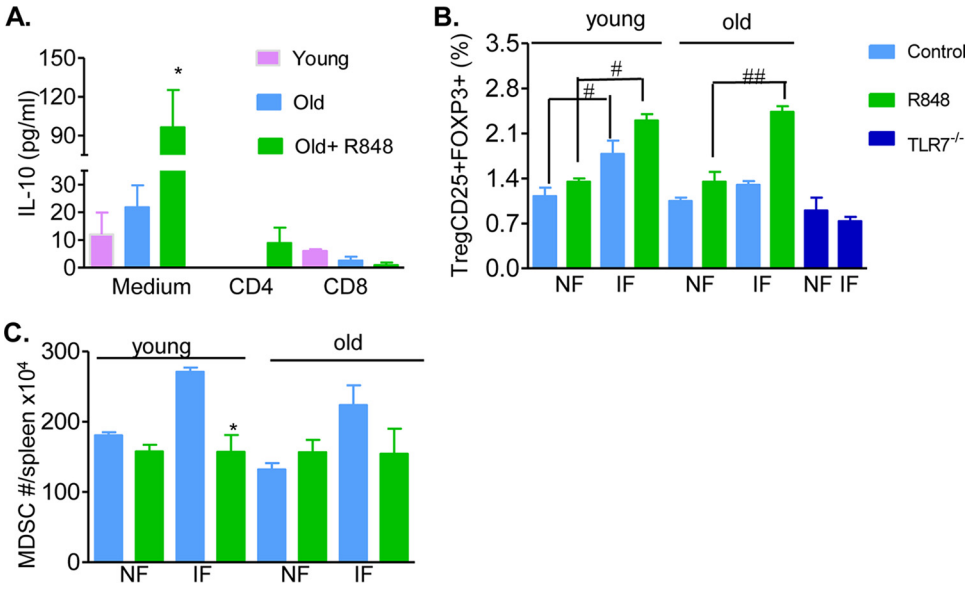

FIG 8 IL-10, Treg, and MDSC responses in R848-treated young and old mice during NS4B-P38G mutant infection. (A) Splenocytes were harvested on day 8 after primary WNV NS4B-P38G infection. IL-10 production in culture supernatants of splenocytes cultured alone or with WNV-specific peptides for $CD4^+$ or $CD8^+$ T cells is shown. (B) Treg expansion. Splenocytes from naive or NS4B-P38G-infected young wild-type and $TLR7^{-/-}$ mice and old mice with or without R848 treatment were stained for CD4, Foxp3, and CD25. (C) MDSC response. Splenocytes from naive or NS4B-P38G-infected young and old mice on day 7 with or without R848 treatment were stained for CD11b and Gr1. *, $P < 0.05$ (compared to mock group); #, $P < 0.05$; ##, $P < 0.01$ (compared to noninfected group).

able to allow a conclusion (43). Another study showed that vaccination with a live, single-cycle virus vaccine, RepliVAX WN, induced significantly lower primary and memory T and B cell responses in old mice (44). We have previously demonstrated that the WNV NS4B-P38G mutant is highly attenuated in young adult mice and has the ability to induce a robust protective immune response, both of which are features of an ideal vaccine candidate (11, 17). In this study, we found that WNV NS4B-P38G-immunized old mice developed levels of protective memory responses similar to those in young mice upon infection with the mutant but were susceptible to clinical disease following infection with the virus. This raises concerns about the safety rather than the efficacy of utilizing live attenuated vaccine candidates such as this one and led us to investigate the mechanism of age-related susceptibility. We demonstrated that an age-associated TLR7 dysregulation partially contributes to innate and adaptive T cell compromise and enhances host susceptibility to WNV NS4B mutant infection. Consistent with our previous findings that memory T cell development is mediated by TLR7-independent MyD88 signaling during NS4B-P38G mutant infection (17), NS4B-P38G mutant-infected old mice developed levels of protective memory T cell responses similar to those in young mice. Furthermore, our results showed that R848 treatment rescued the TLR7-mediated immune compromise and ultimately decreased host vulnerability in old mice during NS4B-P38G mutant infection.

Age-associated dysregulation of TLR signaling has been found to contribute to the increased morbidity and mortality from infectious diseases found in geriatric patients (45, 46). Here, TLR7 dysregulation was associated with impaired responses of γδ T cells, DCs, Th1 cells, and Tregs in WNV NS4B-P38G mutant-infected old mice (Fig. 9). γδ T cells are important innate lymphocytes that respond quickly in control of virus dissemination upon WNV infection (31, 47). Besides TCRs, they express multiple types of TLRs, which provide costimulatory signals during TCR activation of the cells (39, 48). TLR7 is required for γδ T cell expansion in WNV-infected young mice. A reduced costimulatory effect of TLR7 on γδ T cells of old mice upon *in vitro* anti-CD3 stimulation was previously reported (39). In this study, we found that γδ T cell expansion was delayed and reduced in old mice in response to NS4B-P38G mutant infection compared to that in young mice. Significantly, this compromise could be rescued by activation of TLR7 with R848 treatment. Dysregulated TLR7 signaling also led to lower levels of type I IFNs and inflammatory cytokines and reduced APC functions in DCs of old mice. TLR7 signaling is involved in T cell activation in NS4B-P38G mutant-infected young mice via promotion of DC maturation and activation (17, 49). Thus, impaired DC functions in old mice could lead to lower effector T cell responses during NS4B mutant

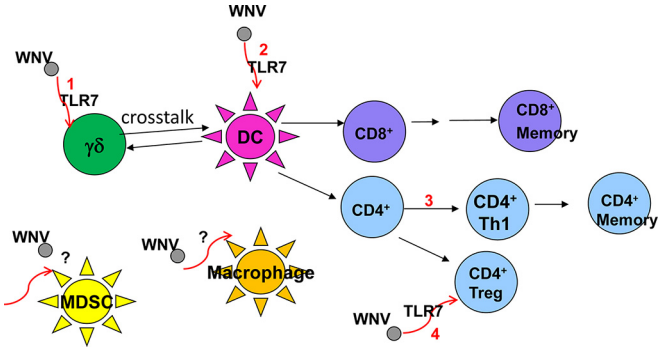

FIG 9 Effect of dysregulation of TLR7 on host immunity against WNV NS4B-P38G mutant-infected old mice. 1, impaired γδ T cell expansion; 2, reduced DC activation and maturation; 3, suppression of T helper 1 response indirectly via DC maturation and/or γδ T cell-DC cross talk; 4, impaired Treg expansion.

infection. Furthermore, γδ T cell compromise also contributed to reduced effector T cell responses, as these cells promote DC maturation via cross talk during WNV infection (50). TLR-activated DCs enhanced $CD4^+$ T cell responses in old mice via cytokine-dependent mechanisms (51). Our results showed that R848 induced a robust inflammatory cytokine response and higher MHC II expression in old DCs, which in turn triggered more T cell proliferation during *in vitro* coculture. Finally, dysregulation of TLR7 impaired Treg function in NS4B-P38G-infected old mice. Higher levels of peripheral $CD4^+$ $CD25^+$ $Foxp3^+$ "natural" Tregs after infection protect young adult mice against severe WNV disease (52). Although aging is associated with increased Treg function (40, 53), we noted lower number of Tregs in old mice than in young mice upon WNV NS4B-P38G mutant infection. $CD4^+$ Treg expansion was also compromised in NS4B-P38G-infected $TLR7^{-/-}$ mice. Thus, a TLR7-mediated Treg dysfunction in old mice partially contributes to an enhanced vulnerability to NS4B-P38G infection.

Conflicting results regarding the role of TLR7 signaling in regulating Treg functions have been reported. In an experimental autoimmune encephalomyelitis model, an inhibitory effect of TLR7 signaling on the generation and function of Tregs was demonstrated (54), which was presumably via TLR7 activation of DCs to reduce Foxp3 expression and suppress functions of Tregs (55). Others have shown that activating TLR7 signaling enhanced Treg functions. For example, R848 induced Treg-mediated suppression of asthma disease in mice (56). Additionally, TLR7 stimulation enhanced the ability of Tregs to suppress T cell proliferation upon TCR activation (57). The authors of these studies proposed that activation of TLR7 was due to direct effects on T cells, particularly Tregs. In fact, TLR7 mRNA was reported to be preferentially expressed on $CD4^+$ $CD25^+$ Tregs compared with $CD4^+$ $CD25^-$ T cells (58). In agreement with these results, we found that R848 increased $Foxp3^+$ Tregs in old mice during WNV NS4B-P38G mutant infection.

In this study, a dysregulation of either TLR3 and/or TLR7 partially contributes to the blunted type I IFN responses in old DCs (Fig. 3). This may explain why no significant changes in serum type I IFN responses were observed in NS4B-P38G mutant-infected old mice despite higher viral loads in these mice than in young mice. Here, old mice produced higher levels of serum inflammatory cytokines and IL-10 upon infection. This seems to be independent of TLR7 dysregulation, as similar levels of innate cytokine production were noted in R848-treated old mice during NS4B-P38G mutant infection. Furthermore, CL097 treatment and the WNV NS4B-P38G mutant induced a differential innate cytokine response in macrophages of old mice (Fig. 4), which also indicates a TLR7-independent innate signaling pathway may play a dominant role upon NS4B-P38G infection. Although dysregulated TLR3 in WNV-infected macrophages of elderly donors has been reported to result in elevated cytokine levels (59), we observed the same levels of inflammatory cytokine responses in poly(I·C)-treated young and old macrophages. Additionally, during influenza virus infection, TLR7 sensing decreased the number of MDSCs by promoting differentiation of these cells into other activated myeloid cell types (60). Likewise, we found that R848 reduced the MDSC number upon NS4B-P38G mutant infection in young mice but not in old mice. Thus, involvement of other PRRs may contribute to a lower innate cytokine response in macrophages and/or other myeloid cells of old mice during WNV infection.

During microbial infection, the production of type I IFNs and inflammatory cytokines induced by the recognition of PRRs via their cognate ligands is critical for control of replication, enhancement of APC functions, and influencing both primary and secondary $CD4^+$ and $CD8^+$ T cell responses (61–64). They can also act directly on T cells to modulate cell proliferation, survival, and/or differentiation into effector cells both *in vitro* and *in vivo* (65–68). The use of several PRR agonists in various established vaccines in order to boost the protective efficacy has been documented (69–71). Small-molecule TLR7/8 agonists have been evaluated as vaccine adjuvants, including resquimod, imiquimod, and similar analogs. TLR7/8 agonist adjuvants promote antigen acquisition and presentation by DCs (72) by themselves or act synergistically with other TLRs (73). This will further induce Th-1-like cells (IFN-γ-producing $CD4^+$ T cells and IgG2-producing B cells), with concomitant inhibition of Th2 immunity in many animal studies. In this study, our results suggest that R848 could rescue the immune cell compromise in old mice and increase the safety of using the NS4B-P38G mutant as a vaccine candidate. Studies that contribute to a mechanistic understanding of immune defects in the elderly may allow the development of strategies to improve responses to infectious diseases and to increase vaccine efficacy and safety in aging individuals.

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REFERENCES

1. **Campbell GL, Marfin AA, Lanciotti RS, Gubler DJ.** 2002. West Nile virus. Lancet Infect Dis **2:**519–529. http://dx.doi.org/10.1016/S1473-3099(02)00368-7.
2. **Solomon T, Ooi MH, Beasley DW, Mallewa M.** 2003. West Nile encephalitis. BMJ **326:**865–869. http://dx.doi.org/10.1136/bmj.326.7394.865.
3. **Wang T, Fikrig E.** 2004. Immunity to West Nile virus. Curr Opin Immunol **16:**519–523. http://dx.doi.org/10.1016/j.coi.2004.05.008.
4. **Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, Garmendia AE, Van Kruiningen HJ.** 1999. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. Science **286:**2331–2333. http://dx.doi.org/10.1126/science.286.5448.2331.
5. **Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, Crise B, Volpe KE, Crabtree MB, Scherret JH, Hall RA, MacKenzie JS, Cropp CB, Panigrahy B, Ostlund E, Schmitt B, Malkinson M, Banet C, Weissman J, Komar N, Savage HM, Stone W, McNamara T, Gubler DJ.** 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science **286:**2333–2337. http://dx.doi.org/10.1126/science.286.5448.2333.
6. **Munoz-Jordan JL, Laurent-Rolle M, Ashour J, Martinez-Sobrido L, Ashok M, Lipkin WI, Garcia-Sastre A.** 2005. Inhibition of alpha/beta

interferon signaling by the NS4B protein of flaviviruses. J Virol **79:**8004–8013. http://dx.doi.org/10.1128/JVI.79.13.8004-8013.2005.

7. **Liu WJ, Wang XJ, Mokhonov VV, Shi PY, Randall R, Khromykh AA.** 2005. Inhibition of interferon signaling by the New York 99 strain and Kunjin subtype of West Nile virus involves blockage of STAT1 and STAT2 activation by nonstructural proteins. J Virol **79:**1934–1942. http://dx.doi.org/10.1128/JVI.79.3.1934-1942.2005.
8. **Evans JD, Seeger C.** 2007. Differential effects of mutations in NS4B on West Nile virus replication and inhibition of interferon signaling. J Virol **81:**11809–11816. http://dx.doi.org/10.1128/JVI.00791-07.
9. **Munoz-Jordan JL, Sanchez-Burgos GG, Laurent-Rolle M, Garcia-Sastre A.** 2003. Inhibition of interferon signaling by dengue virus. Proc Natl Acad Sci U S A **100:**14333–14338. http://dx.doi.org/10.1073/pnas.2335168100.
10. **Wicker JA, Whiteman MC, Beasley DW, Davis CT, McGee CE, Lee JC, Higgs S, Kinney RM, Huang CY, Barrett AD.** 2012. Mutational analysis of the West Nile virus NS4B protein. Virology **426:**22–33. http://dx.doi.org/10.1016/j.virol.2011.11.022.
11. **Welte T, Xie G, Wicker JA, Whiteman MC, Li L, Rachamallu A, Barrett A, Wang T.** 2011. Immune responses to an attenuated West Nile virus NS4B-P38G mutant strain. Vaccine **29:**4853–4861. http://dx.doi.org/10.1016/j.vaccine.2011.04.057.
12. **Xie G, Luo H, Tian B, Mann B, Bao X, McBride J, Tesh R, Barrett AD, Wang T.** 2015. A West Nile virus NS4B-P38G mutant strain induces cell intrinsic innate cytokine responses in human monocytic and macrophage cells. Vaccine **33:**869–878. http://dx.doi.org/10.1016/j.vaccine.2014.12.056.
13. **Brien JD, Uhrlaub JL, Hirsch A, Wiley CA, Nikolich-Zugich J.** 2009. Key role of T cell defects in age-related vulnerability to West Nile virus. J Exp Med **206:**2735–2745. http://dx.doi.org/10.1084/jem.20090222.
14. **Welte T, Lamb J, Anderson JF, Born WK, O'Brien RL, Wang T.** 2008. Role of two distinct gammadelta T cell subsets during West Nile virus infection. FEMS Immunol Med Microbiol **53:**275–283. http://dx.doi.org/10.1111/j.1574-695X.2008.00430.x.
15. **Town T, Bai F, Wang T, Kaplan AT, Qian F, Montgomery RR, Anderson JF, Flavell RA, Fikrig E.** 2009. Toll-like receptor 7 mitigates lethal West Nile encephalitis via interleukin 23-dependent immune cell infiltration and homing. Immunity **30:**242–253. http://dx.doi.org/10.1016/j.immuni.2008.11.012.
16. **Welte T, Reagan K, Fang H, Machain-Williams C, Zheng X, Mendell N, Chang GJ, Wu P, Blair CD, Wang T.** 2009. Toll-like receptor 7-induced immune response to cutaneous West Nile virus infection. J Gen Virol **90:**2660–2668. http://dx.doi.org/10.1099/vir.0.011783-0.
17. **Xie G, Welte T, Wang J, Whiteman MC, Wicker JA, Saxena V, Cong Y, Barrett AD, Wang T.** 2013. A West Nile virus NS4B-P38G mutant strain induces adaptive immunity via TLR7-MyD88-dependent and independent signaling pathways. Vaccine **31:**4143–4151. http://dx.doi.org/10.1016/j.vaccine.2013.06.093.
18. **Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, Akira S.** 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol **3:**196–200. http://dx.doi.org/10.1038/ni758.
19. **Daffis S, Samuel MA, Suthar MS, Keller BC, Gale M, Jr, Diamond MS.** 2008. Interferon regulatory factor IRF-7 induces the antiviral alpha interferon response and protects against lethal West Nile virus infection. J Virol **82:**8465–8475. http://dx.doi.org/10.1128/JVI.00918-08.
20. **Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, Komar N, Panella NA, Allen BC, Volpe KE, Davis BS, Roehrig JT.** 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol **38:**4066–4071.
21. **Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA.** 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med **10:**1366–1373. http://dx.doi.org/10.1038/nm1140.
22. **Edwards AD, Diebold SS, Slack EM, Tomizawa H, Hemmi H, Kaisho T, Akira S, Reis e Sousa C.** 2003. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. Eur J Immunol **33:**827–833. http://dx.doi.org/10.1002/eji.200323797.
23. **Welte T, Aronson J, Gong B, Rachamallu A, Mendell N, Tesh R, Paessler S, Born WK, O'Brien RL, Wang T.** 2011. Vgamma4+ T cells regulate host immune response to West Nile virus infection. FEMS Immunol Med Microbiol **63:**183–192. http://dx.doi.org/10.1111/j.1574-695X.2011.00840.x.
24. **Wang S, Welte T, McGargill M, Town T, Thompson J, Anderson JF, Flavell RA, Fikrig E, Hedrick SM, Wang T.** 2008. Drak2 contributes to West Nile virus entry into the brain and lethal encephalitis. J Immunol **181:**2084–2091. http://dx.doi.org/10.4049/jimmunol.181.3.2084.
25. **Brien JD, Uhrlaub JL, Nikolich-Zugich J.** 2008. West Nile virus-specific CD4 T cells exhibit direct antiviral cytokine secretion and cytotoxicity and are sufficient for antiviral protection. J Immunol **181:**8568–8575. http://dx.doi.org/10.4049/jimmunol.181.12.8568.
26. **Brien JD, Uhrlaub JL, Nikolich-Zugich J.** 2007. Protective capacity and epitope specificity of CD8(+) T cells responding to lethal West Nile virus infection. Eur J Immunol **37:**1855–1863. http://dx.doi.org/10.1002/eji.200737196.
27. **Purtha WE, Myers N, Mitaksov V, Sitati E, Connolly J, Fremont DH, Hansen TH, Diamond MS.** 2007. Antigen-specific cytotoxic T lymphocytes protect against lethal West Nile virus encephalitis. Eur J Immunol **37:**1845–1854. http://dx.doi.org/10.1002/eji.200737192.
28. **Samuel MA, Diamond MS.** 2005. Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. J Virol **79:**13350–13361. http://dx.doi.org/10.1128/JVI.79.21.13350-13361.2005.
29. **Bai F, Town T, Qian F, Wang P, Kamanaka M, Connolly TM, Gate D, Montgomery RR, Flavell RA, Fikrig E.** 2009. IL-10 signaling blockade controls murine West Nile virus infection. PLoS Pathog **5:**e1000610. http://dx.doi.org/10.1371/journal.ppat.1000610.
30. **Schneider BS, McGee CE, Jordan JM, Stevenson HL, Soong L, Higgs S.** 2007. Prior exposure to uninfected mosquitoes enhances mortality in naturally-transmitted West Nile virus infection. PLoS One **2:**e1171. http://dx.doi.org/10.1371/journal.pone.0001171.
31. **Wang T, Scully E, Yin Z, Kim JH, Wang S, Yan J, Mamula M, Anderson JF, Craft J, Fikrig E.** 2003. IFN-gamma-producing gammadelta T cells help control murine West Nile virus infection. J Immunol **171:**2524–2531. http://dx.doi.org/10.4049/jimmunol.171.5.2524.
32. **Roehrig JT, Staudinger LA, Hunt AR, Mathews JH, Blair CD.** 2001. Antibody prophylaxis and therapy for flavivirus encephalitis infections. Ann N Y Acad Sci **951:**286–297.
33. **Diamond MS, Shrestha B, Marri A, Mahan D, Engle M.** 2003. B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. J Virol **77:**2578–2586. http://dx.doi.org/10.1128/JVI.77.4.2578-2586.2003.
34. **Shrestha B, Samuel MA, Diamond MS.** 2006. CD8+ T cells require perforin to clear West Nile virus from infected neurons. J Virol **80:**119–129. http://dx.doi.org/10.1128/JVI.80.1.119-129.2006.
35. **Wang Y, Lobigs M, Lee E, Mullbacher A.** 2003. CD8+ T cells mediate recovery and immunopathology in West Nile virus encephalitis. J Virol **77:**13323–13334. http://dx.doi.org/10.1128/JVI.77.24.13323-13334.2003.
36. **Qian F, Wang X, Zhang L, Lin A, Zhao H, Fikrig E, Montgomery RR.** 2011. Impaired interferon signaling in dendritic cells from older donors infected in vitro with West Nile virus. J Infect Dis **203:**1415–1424. http://dx.doi.org/10.1093/infdis/jir048.
37. **Fredericksen BL, Gale M, Jr.** 2006. West Nile virus evades activation of interferon regulatory factor 3 through RIG-I-dependent and -independent pathways without antagonizing host defense signaling. J Virol **80:**2913–2923. http://dx.doi.org/10.1128/JVI.80.6.2913-2923.2006.
38. **Daffis S, Samuel MA, Suthar MS, Gale M, Jr, Diamond MS.** 2008. Toll-like receptor 3 has a protective role against West Nile virus infection. J Virol **82:**10349–10358. http://dx.doi.org/10.1128/JVI.00935-08.
39. **Zhang J, Wang J, Pang L, Xie G, Welte T, Saxena V, Wicker J, Mann B, Soong L, Barrett A, Born W, O'Brien R, Wang T.** 2014. The co-stimulatory effects of MyD88-dependent Toll-like receptor signaling on activation of murine gammadelta T cells. PLoS One **9:**e108156. http://dx.doi.org/10.1371/journal.pone.0108156.
40. **Garg SK, Delaney C, Toubai T, Ghosh A, Reddy P, Banerjee R, Yung R.** 2014. Aging is associated with increased regulatory T-cell function. Aging Cell **13:**441–448. http://dx.doi.org/10.1111/acel.12191.
41. **Verschoor CP, Johnstone J, Millar J, Dorrington MG, Habibagahi M, Lelic A, Loeb M, Bramson JL, Bowdish DM.** 2013. Blood CD33(+)HLA-DR(−) myeloid-derived suppressor cells are increased with age and a history of cancer. J Leukoc Biol **93:**633–637. http://dx.doi.org/10.1189/jlb.0912461.
42. **Jeisy-Scott V, Kim JH, Davis WG, Cao W, Katz JM, Sambhara S.** 2012. TLR7 recognition is dispensable for influenza virus A infection, but im-

portant for the induction of hemagglutinin-specific antibodies in response to the 2009 pandemic split vaccine in mice. J Virol **86:**10988–10998. http://dx.doi.org/10.1128/JVI.01064-12.
43. **Beasley DW.** 2011. Safety and immunogenicity of a chimeric vaccine for West Nile virus in aged subjects. Expert Rev Vaccines **10:**601–604. http://dx.doi.org/10.1586/erv.11.47.
44. **Uhrlaub JL, Brien JD, Widman DG, Mason PW, Nikolich-Zugich J.** 2011. Repeated in vivo stimulation of T and B cell responses in old mice generates protective immunity against lethal West Nile virus encephalitis. J Immunol **186:**3882–3891. http://dx.doi.org/10.4049/jimmunol.1002799.
45. **Panda A, Qian F, Mohanty S, van Duin D, Newman FK, Zhang L, Chen S, Towle V, Belshe RB, Fikrig E, Allore HG, Montgomery RR, Shaw AC.** 2010. Age-associated decrease in TLR function in primary human dendritic cells predicts influenza vaccine response. J Immunol **184:**2518–2527. http://dx.doi.org/10.4049/jimmunol.0901022.
46. **Shaw AC, Panda A, Joshi SR, Qian F, Allore HG, Montgomery RR.** 2011. Dysregulation of human Toll-like receptor function in aging. Ageing Res Rev **10:**346–353. http://dx.doi.org/10.1016/j.arr.2010.10.007.
47. **Wang T, Welte T, Saxena V, Xie G.** 2011. Immunity versus immunopathology in West Nile virus induced encephalitis. *In* Ruzek D (ed), Flavivirus encephalitis InTech. http://dx.doi.org/10.5772/20355.
48. **Wesch D, Beetz S, Oberg HH, Marget M, Krengel K, Kabelitz D.** 2006. Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes. J Immunol **176:**1348–1354. http://dx.doi.org/10.4049/jimmunol.176.3.1348.
49. **Xie G, Whiteman MC, Wicker JA, Barrett AD, Wang T.** 2014. In vitro analysis of MyD88-mediated cellular immune response to West Nile virus mutant strain infection. J Vis Exp http://dx.doi.org/10.3791/52121:e52121.
50. **Fang H, Welte T, Zheng X, Chang GJ, Holbrook MR, Soong L, Wang T.** 2010. Gammadelta T cells promote the maturation of dendritic cells during West Nile virus infection. FEMS Immunol Med Microbiol **59:**71–80. http://dx.doi.org/10.1111/j.1574-695X.2010.00663.x.
51. **Jones SC, Brahmakshatriya V, Huston G, Dibble J, Swain SL.** 2010. TLR-activated dendritic cells enhance the response of aged naive CD4 T cells via an IL-6-dependent mechanism. J Immunol **185:**6783–6794. http://dx.doi.org/10.4049/jimmunol.0901296.
52. **Lanteri MC, O'Brien KM, Purtha WE, Cameron MJ, Lund JM, Owen RE, Heitman JW, Custer B, Hirschkorn DF, Tobler LH, Kiely N, Prince HE, Ndhlovu LC, Nixon DF, Kamel HT, Kelvin DJ, Busch MP, Rudensky AY, Diamond MS, Norris PJ.** 2009. Tregs control the development of symptomatic West Nile virus infection in humans and mice. J Clin Invest **119:**3266–3277. http://dx.doi.org/10.1172/JCI39387.
53. **Williams-Bey Y, Jiang J, Murasko DM.** 2011. Expansion of regulatory T cells in aged mice following influenza infection. Mech Ageing Dev **132:**163–170. http://dx.doi.org/10.1016/j.mad.2011.03.001.
54. **Lalive PH, Benkhoucha M, Tran NL, Kreutzfeldt M, Merkler D, Santiago-Raber ML.** 2014. TLR7 signaling exacerbates CNS autoimmunity through downregulation of Foxp3+ Treg cells. Eur J Immunol **44:**46–57. http://dx.doi.org/10.1002/eji.201242985.
55. **Hackl D, Loschko J, Sparwasser T, Reindl W, Krug AB.** 2011. Activation of dendritic cells via TLR7 reduces Foxp3 expression and suppressive function in induced Tregs. Eur J Immunol **41:**1334–1343. http://dx.doi.org/10.1002/eji.201041014.
56. **Van LP, Bardel E, Gregoire S, Vanoirbeek J, Schneider E, Dy M, Thieblemont N.** 2011. Treatment with the TLR7 agonist R848 induces regulatory T-cell-mediated suppression of established asthma symptoms. Eur J Immunol **41:**1992–1999. http://dx.doi.org/10.1002/eji.201040914.
57. **Forward NA, Furlong SJ, Yang Y, Lin TJ, Hoskin DW.** 2010. Signaling through TLR7 enhances the immunosuppressive activity of murine CD4+CD25+ T regulatory cells. J Leukoc Biol **87:**117–125. http://dx.doi.org/10.1189/jlb.0908559.
58. **Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, Demengeot J.** 2003. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J Exp Med **197:**403–411. http://dx.doi.org/10.1084/jem.20021633.
59. **Kong KF, Delroux K, Wang X, Qian F, Arjona A, Malawista SE, Fikrig E, Montgomery RR.** 2008. Dysregulation of TLR3 impairs the innate immune response to West Nile virus in the elderly. J Virol **82:**7613–7623. http://dx.doi.org/10.1128/JVI.00618-08.
60. **Jeisy-Scott V, Davis WG, Patel JR, Bowzard JB, Shieh WJ, Zaki SR, Katz JM, Sambhara S.** 2011. Increased MDSC accumulation and Th2 biased response to influenza A virus infection in the absence of TLR7 in mice. PLoS One **6:**e25242. http://dx.doi.org/10.1371/journal.pone.0025242.
61. **Pham NL, Badovinac VP, Harty JT.** 2009. A default pathway of memory CD8 T cell differentiation after dendritic cell immunization is deflected by encounter with inflammatory cytokines during antigen-driven proliferation. J Immunol **183:**2337–2348. http://dx.doi.org/10.4049/jimmunol.0901203.
62. **Chandran SS, Verhoeven D, Teijaro JR, Fenton MJ, Farber DL.** 2009. TLR2 engagement on dendritic cells promotes high frequency effector and memory CD4 T cell responses. J Immunol **183:**7832–7841. http://dx.doi.org/10.4049/jimmunol.0901683.
63. **Conti L, Casetti R, Cardone M, Varano B, Martino A, Belardelli F, Poccia F, Gessani S.** 2005. Reciprocal activating interaction between dendritic cells and pamidronate-stimulated gammadelta T cells: role of CD86 and inflammatory cytokines. J Immunol **174:**252–260. http://dx.doi.org/10.4049/jimmunol.174.1.252.
64. **Dieli F, Caccamo N, Meraviglia S, Ivanyi J, Sireci G, Bonanno CT, Ferlazzo V, La Mendola C, Salerno A.** 2004. Reciprocal stimulation of gammadelta T cells and dendritic cells during the anti-mycobacterial immune response. Eur J Immunol **34:**3227–3235. http://dx.doi.org/10.1002/eji.200425368.
65. **Badovinac VP, Tvinnereim AR, Harty JT.** 2000. Regulation of antigen-specific CD8+ T cell homeostasis by perforin and interferon-gamma. Science **290:**1354–1358. http://dx.doi.org/10.1126/science.290.5495.1354.
66. **Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF.** 2005. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. J Immunol **174:**4465–4469. http://dx.doi.org/10.4049/jimmunol.174.8.4465.
67. **Xiao Z, Casey KA, Jameson SC, Curtsinger JM, Mescher MF.** 2009. Programming for CD8 T cell memory development requires IL-12 or type I IFN. J Immunol **182:**2786–2794. http://dx.doi.org/10.4049/jimmunol.0803484.
68. **Mescher MF, Curtsinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, Popescu F, Xiao Z.** 2006. Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev **211:**81–92. http://dx.doi.org/10.1111/j.0105-2896.2006.00382.x.
69. **Zimmermann S, Dalpke A, Heeg K.** 2008. CpG oligonucleotides as adjuvant in therapeutic vaccines against parasitic infections. Int J Med Microbiol **298:**39–44. http://dx.doi.org/10.1016/j.ijmm.2007.07.011.
70. **Alexopoulou L, Thomas V, Schnare M, Lobet Y, Anguita J, Schoen RT, Medzhitov R, Fikrig E, Flavell RA.** 2002. Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in TLR1- and TLR2-deficient mice. Nat Med **8:**878–884.
71. **Lahiri A, Das P, Chakravortty D.** 2008. Engagement of TLR signaling as adjuvant: towards smarter vaccine and beyond. Vaccine **26:**6777–6783. http://dx.doi.org/10.1016/j.vaccine.2008.09.045.
72. **Kastenmuller K, Wille-Reece U, Lindsay RW, Trager LR, Darrah PA, Flynn BJ, Becker MR, Udey MC, Clausen BE, Igyarto BZ, Kaplan DH, Kastenmuller W, Germain RN, Seder RA.** 2011. Protective T cell immunity in mice following protein-TLR7/8 agonist-conjugate immunization requires aggregation, type I IFN, and multiple DC subsets. J Clin Invest **121:**1782–1796. http://dx.doi.org/10.1172/JCI45416.
73. **Vasilakos JP, Tomai MA.** 2013. The use of Toll-like receptor 7/8 agonists as vaccine adjuvants. Expert Rev Vaccines **12:**809–819. http://dx.doi.org/10.1586/14760584.2013.811208.